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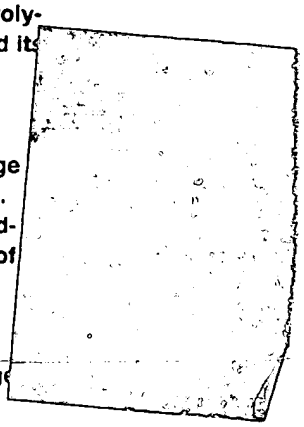

Description

The present invention relates to a medicament comprising a complexe f copper ion, with such fractions of heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, heparin fragments, heparan sulfate fragments, and oligosaccharides derived from heparin or heparan sulfate that specifically bind to the copper ion, or a salt of such fractions, to a process for obtaining such metal-binding fractions and metal complexes thereof, and to the use of such complexes in therapy. It was unexpectedly found that the inhibitory properties on angiogenesis of these novel complexes of the invention in vivo in presence of a suitable angiostatic agent, especially a steroid were markedly enhanced with respect to such fractions that were not bound to metal ions and also with respect to the unfractionated products.

Background of the invention

It has been shown that a combination of heparin or a hexasaccharide fragment together with a suitable steroid cause inhibition of angiogenesis in mammals and that tumor masses in mammals are caused to regress and metastasis is prevented (Folkman et al.; Science 1983, 221, 719-725). It was also shown that this combination was effectively inhibiting other angiogenesis depending processes such as fertilization of the rat (Folkman et al., European Patent Application EP-0114589). It is also effective in reducing osteoporosis and in treating deseases involving neovascularization, such as neovascular diseases of the eye. Also because of the occurrence of angiogenesis in psoriasis and arthritis, it is expected that this combination will be useful in treating those diseases.

Whereas further research and development with respect to the steroid part of this combination that inhibits angiogenesis has yielded more potent and specific steroids (Crum et al., Science 1985, 230, 1375-1378), no such progress has been made with regards to the heparin component of the heparin steroid composition. A synthetic highly sulfated non-anticoagulant pentasaccharide was recently shown to possess inhibiting effect on angiogenesis in the presence of a steroid to the same extent as its highly anticoagulant active analogue (the 3-0-sulfated analogue) (Choay et al., European Patent Application EP-0140781). This finding further supports the finding that the anticoagoulant (anti-Xa) properties of heparin and heparin fragments are not required for the heparin and heparin fragments to be inhibitory on angiogenesis in the presence of steroids (Folkman et al., Science 1983, 221, 719-725; Crum et al., Science 1985, 230, 1375-1378).

Heparin is a glycosidically linked highly sulfated copolymer of uronic acids and D-glucosamine. The uronic acids being L-iduronic acid or D-glucuronic acid of which the former usually is sulfated and the latter usually nonsulfated. The glucosamine is either N-sulfated or N-acetylated and also frequently 6-0-sulfated. Small amounts of other structural variants also occur. The exact structure of heparin and the precise nature of its antithrombotic mechanism of action has not been elucidated although it has been in widespread use for almost 50 years. Heparin is polydisperse with a molecular weight range from 3,000-30,000 with many structural variations within a given chain. The exact composition of heparin varies depending on its source, which usually is porcine intestinal mucosa, bovine lung, bovine intestinal mucosa, or ovine intestinal mucosa and also depending on the method for its preparation and purification,. Low molecular weight heparin (molecular weight range 2,000-10,000) has been isolated in small amounts by fractionation of standard heparin. Heparin fragments of molecular weight range 500-10,000 has been prepared by partial de-polymerization of heparin by chemical or enzymatic methods. Chemical depolymerization has been carried out in many different ways, frequently by nitrites at low pH, by alkaline β-elimination usually after esterification of uronic acids or by oxidative methods usually using peroxides or periodate. After de-polymerization with nitrites the newly formed anhydromannose at the reducing end of the heparin fragments and the oligosaccharides derived from such a fragment usually are reduced to anhydromannitol or oxidized to anhydromannonic acid for increased stability of the product. The enzymatic depolymerization and the alkaline β-elimination results in the same 4,5-unsaturation in the nonreducing end of the heparin fragments and in the oligosaccharides derived from these fragments. For increased stability such unsaturated groups can subsequently be reduced by standard procedures for example catalytic hydrogenation, or the whole 4,5-unsaturated monosaccharide may be eliminated by for example mild acid treatment or by applying metal containing reagents such as mercury salts. In the latter case heparin fragments, heparan sulfat fragments, and oligosaccharides derived from them containing an uneven number of saccharide moieties are obtained. Heparan sulfate is the only other glycosaminoglycan besides heparin that also contains N-sulfated glucosamines. Most heparan sulfate however contains more N-acetylated glucosamin than N-sulfated glucosamine, the opposite being the case for heparin.

The same methods of fractionation and depolymerization used for heparin are also applicable to heparan sulfate. The enzyme used for heparan sulfate is usually a hepartitinase (heparanase) instead of heparinase which is most commonly used for heparin. Small amounts of heparan sulfate is usually found in standard heparins. Heparan sulfate also constitutes a large part of heparin by-products particularly form bovine lung.

Heparin by itself (without a steroid) enhances the intensity of angiogenesis induced by tumors and by tumor derived factors in vivo, although in the absence of tumor cells or tumor extracts or tumor derived factors neither heparin nor the mast cells which release heparin could induce angiogenesis (Taylor and Folkman, Nature 1982, 297, 307-312).

Some angiogenic factors from normal cells and tissue, for example so-called heparin binding growth factors can also induce angiogenesis and stimulate the growth of capillary endothelial cells. With some growth factors, this stimulation of capillary endothelial cell growth is potentiated by heparin.

Heparin, by virtue of its high negative charge, has a strong affinity for cations, where the binding generally is ionic since pH dependency is usually observed. Clinically used standard heparin is either the sodium or calcium salt of heparinic acid. The calcium heparin usually has a calcium content of about 11 w/w % which corresponds to about 2.8 µmole Ca^{2+}/mg heparin. For cupric ions (Cu^{2+}), binding to heparin was shown to be pH-dependent and for a typical heparin (molecular weight 13100, anticoagulant activity 146 IU/mg) copper binding was 0.606 µmole Cu^{2+}/mg heparin at neutral pH (Stivala SS, Fed. Proc. Fed. Am. Soc. Exp. Bio. 1977, 36, 83-88). Thus cupric ions bind to a lesser extent to heparin than calcium ions at neutral pH, showing for cupric ions about 20% of the binding of that of the calcium ions. Heparin containing 1 µg of copper per 20 µg of heparin (which corresponds to 0.787 µmole Cu^{2+}/mg heparin or about 10 µmole Cu^{2+}/µmole heparin) has been shown to be able to induce angiogenesis in vivo, which heparin by itself could not do (Alessandri G, Raju K, Gullino P M, Microcirculation, Endothelium and Lymphatics 1984, 1, 329-346).

Metal chelate affinity chromatography, also called ligand exchange chromatography or immobilized metal ion adsorption, is usually carried out by binding (chelating) various metal ions, such as for example Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+} and Fe^{3+} to a solid matrix, for example a cation exchange resin or a special metal ion chelator such as Chelating Sepharose® 6B Pharmacia and Chelex™ 100 Bio-Rad and then carry out fractionation of complex mixtures.

Description of the invention

It has been found according to the present invention that complexes of copper ions with such fractions of heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, heparin fragments, heparan sulfate fragments, and oligosaccharides derived from heparin or from heparan sulfate, or a salt of such fractions which fractions specifically bind to the copper ions, exhibit markedly enhanced anti-angiogenic properties when used in conjunction with an angiostatic component, especially a steroid as disclosed above. The present invention accordingly relates to medicament for the treatment of an ailment where reduced angiogenesis is desired, comprising a complex of

(a) a metal ion which is copper and

(b) a fraction of heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, heparin fragments, heparan sulfate fragments and oligosaccharides derived from heparin or from heparan sulfate, or a salt of such fractions, said fraction having a molecular weights from 500 to 35.000 and said salt being a physiologically acceptable salts such as sodium, calcium or ammonium salts,

which fractions bind to the said metal ion and

said complex containing from 5 - 1,000, preferably 10 to 1.000 nmole metal per µmole of component (b).

- the medicament comprising a complex as described above wherein component (b) is heparin or a low molecular weight heparin or a heparin fragment or an oligosaccharide derived from heparin, such as tetrasaccharide, a hexasaccharide, an octasaccharide, a decasaccharide, a dodecasaccharide, a tetradesaccharide or a hexadecasaccharide.
- the medicament comprising a complex as described above wherein component (b) is heparan sulfate, low molecular weight heparan sulfate or heparan sulfate fragments or an oligosaccharides derived from heparan sulfate, such as tetrasaccharide, a hexasaccharide, an octasaccharide, a decasaccharide, a dodecasaccharide, a tetrad saccharide or a hexadecasaccharide.
- the medicament comprising a complex as described above wherein the component (b) is in the form of a salt, preferably sodium or a calcium salt.
- a process for the preparation of a complex as described above consisting of adding heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, heparin fragments, a

heparan sulfate fragment and oligosaccharide derived from heparin or from heparan sulfate to a solid matrix containing iminodiacetic acid groups to which a copper ion is bound, separating the fraction of added material that does not bind to the copper ions on the matrix and thereafter desorption of the fraction bound to the matrix.

- use of a complex of
  (a) a metal ion selected from copper, calcium, manganese, iron and zinc ions and
  (b) a fraction of heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, heparin fragments, heparan sulfate fragments and oligosacarides derived from heparin or from heparan sulfate, or a salt of such fractions, said fraction having a molecular weights from 500 to 35.000 and said salt being a physiologically acceptable salts such as sodium, calcium or ammonium salts,
  which fraction bind to the said metal ion and
  said complex containing from 5 to 1.000 nmole metal per µmole of component (b)
  in the preparation of a medicament for the treatment of an ailment, where reduced angiogenesis is desired.
- use as described above in which the medicament comprises the complex in conjunction with an angiostatic component, especially a so-called angiostatic steroid component.

The fractions of this invention contain constituents of heparin and/or heparan sulfate. They are isolated from heparins and heparan sulfates of bovine, porcine, ovine, or other orgin, low molecular weight heparins or heparan sulfates, fragments of heparins and of heparan sulfate, oligosaccharides from any of these products and also such oligosaccharides which are size homogenous and from other fractions containing heparin or heparan sulfate constituents, for example "byproducts" from heparin production. The fractions of the invention will contain from 2 to about 120 sugar moieties corresponding to molecular weights from 500 to about 35,000. With "salts" of the said heparin fragments is meant physiologically acceptable salts such as sodium, calcium or ammonium salts. The sodium and calcium salts, especially the sodium salt are the preferred salts. Normally, the heparin complexes of the invention will be used in the form of a salt thereof.

The fractions of the invention are characterized by that they are retained by copper ions chelated to a solid matrix and in that they can be desorbed from this copper ion-containing solid matrix by a pH-gradient, or a competitive ligand or other chelating agents. They may also be produced in this way from the starting materials described above.

The content of copper ions will be in the range of from 5 to 1,000 nmole per µmole of the heparin/heparan sulfate component. A preferred interval will be from 10 to 1,000 nmole per µmole of the heparin/heparan sulfate component. Among the copper binding fractions of the heparin/heparan sulfate component it will be preferred to use low molecular weight heparin or low molecular weight heparan sulfate having a molecular weight of from 500 to 10,000 or from 500 to 8,000 and low molecular weight oligosaccharides derived from heparin or from heparan sulfate, especially tetrasaccharides, hexasaccharides, octasaccharides, decasaccharides, dodecasaccharides, tetradecasaccharides and hexadecasaccharides.

Angiogenesis, the growth of new capillary blood vessels, is important in normal processes such as development of the embryo, formation of the corpus luteum and wound healing. It is also a component in pathologic processes such as chronic inflammation, certain immune responses and neoplasia. Angiogenesis is also a property of most solid tumours and is necessary for their growth.

Medical indications where reduced angiogenesis is desired may be exemplified with

- tumours, especially solid tumours
- prevention of metastasis
- neovascular diseases of the eye, such as retrolental fibroplasia, diabetic retinopathy, and neovascular glaucoma
- osteoporosis
- psoriasis
- arthritis

The complexes as described above are preferably intended for use in conjunction with an angiostatic component, especially a so-called angiostatic steroid (Crum et al., Science Vol. 230, p. 1377), that is a steroid with high anti-angiogenic effect and low glucocorticoid and mineralo-corticoid effect. Examples of such steroids are given by Crum et al. and include

- cortisone
- hydrocortisone
- 11α-isomer of hydrocortisone
- 6α-fluoro-17,21-dihydroxy-16β-methyl-pregna-4,9,(11)diene-3,20-dione

- 17α-hydroxy-progesterone.
- 5β-pregnane-3α;17α,21-triol-20-one (Tetrahydro-S)
- tetrahydrocortisol or its 3α-glucuronide

In clinical practice, administration of these complexes will be made essentially as is prescribed for standard heparin and for low molecular weight heparin. Thus, topical and parenteral administration - i.v., subcutaneous, intramuscular - are foreseen. Local administration e.g. in the eye or to a tumor may be desired. In addition, the complexes can be administered orally.

The steroid component is administered in a manner which is customary for administration of steroids, such by the oral or parenteral route.

The complexes as described above and the angiostatic component may be mixed together prior to administration or may be administered separately.

The amount to be administered of these complex and of the angiostatic component must be adjusted to the individual need of each patient.

For preparing these complexes, heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, a heparin fragment, a heparan sulfate fragment, or an oligosaccharide derived from heparin of from heparan sulfate, or a salt thereof, is reacted with a copper ion, followed by isolation of the complex thus formed.

The reaction can be carried out by means of an ion exchange resin, especially a resin containing iminodiacetic acid groups, or in solution. The reactants are adapted so that the complex obtained will contain in the case of copper ions from 5 to 1,000 nmole copper per µmole of the heparin/heparan sulfate component. Similar amounts will be desired for the other metal complexes of the invention.

For preparing and isolating the fractions of this invention metal chelate affinity chromatography has been carried out using a solid matrix containing iminodiacetic acid groups for binding (chelating) the metal ions (Chelex™ 100, Bio-Rad or Chelating Sepharose® 6B Pharmacia). By using a solution of cupric chloride ($CuCl_2$) as a source of copper ions the copper ions chelated should be mainly cupric ions (Cu^{2+}). Some of the copper ions may however be present as cuprous ions (Cu^{+}). To a column charged with copper ions the sample to be fractionated was applied dissolved in distilled water, a buffer solution, a salt solution of a buffer-salt solution. The column was then washed with the same solvent which was used to dissolve the sample. Material that was not bound to, or very little bound to the copper Chelex column was collected in this fraction. For desorption of applied products that was bound to (retained by) the column three general methods was used:

1. Desorption by a pH gradient. As it has been shown that heparin copper interaction was dependent on pH, this is a valid alternative.
2. Desorption, by an increasing gradient or isocratically by a competitive ligand such as imidazole, histamine, glycine, aquous ammonia, an ammonium salt, preferably ammonium chloride, or a solution containing metal ions of the same kind, which have been bound to the matrix. The solution of the ligand preferably contains the salt and/or the buffer used in the washing step.
3. Desorption by a chelating agent such as EDTA or EGTA which will strip the metal ions from the gel and cause the elution of all the adsorbed material.

Ligand exchange chromatography using copper ions has been applied to protein, amino acids and nucleotides, but not previously to complex carbohydrates such as heparin or heparan sulfate or products derived from them. When heparin, heparan sulfates or their fragments, low molecular weight products, or the size hologenous oligosaccharides derived from those products or biproducts from heparin manufacturing were fractionated by copper chelate affinity chromotography, it was found that a small part of these products became bound to the copper ions on the matrix. After desorption of these tightly bound fractions they were assayed for inhibition of angiogenesis together with hydrocortison or an other angiostatic steroid, as described by Folkman et al., Science 1983, 221, 719-725, Crum et al., Science 1985, 230, 1375-1378. It was then unexpectedly found that those fractions which had been retained by (bound to) copper ions on the matrix showed higher inhibitory effects on angiogenesis in the presence of a so-called angiostatic steroid than the fractions of these products that was not retained by (bound to) the copper ion containing matrix. (Table 1.)

The fractions retained on the copper Chelex column also showed higher inhibitory effect on angiogenesis in presence of a so-called angiostatic steroid (for example hydrocortison) than the respective starting products which were used for the fractionation. (Table 1.)

Clinically used standard heparin according to the U.S. Pharmacopeia and the British Pharmacopeia must not contain more than 30 ppm of heavy metals. By atomic absorption analysis it was found that in heparin, heparan sulfate and the low molecular weight materials, fragments, fractions and oligosaccharides prepared form heparin and heparan sulfate up to about 10 ppm of these heavy metals was copper.

However, within this 0-10 ppm of copper, no correlation between content of copper and degree of inhibition of angiogenesis in the presence of steroid was obvious.

When the fractions eluted from the copper Chelex column were treated with Chelex containing sodium ions, it was found that the first fraction which was eluted with water, a salt solution, a buffer solution, or a buffer-salt solution always contained less than 20 ppm of copper as determined by atomic absorption spectroscopy. However, the binding fractions which were desorbed as described above, after treatment with Chelex containing sodium ions always contained more copper (see Table 2). This residual amount of copper could only be removed with great difficulty, for example by dialysis against large amounts of a solution of EDTA (see Example 9). It was then unexpectedly found that this residual amount of copper ions in the fractions which had been bound to (retained by) the copper Chelex column was necessary for the improved inhibition of angiogenesis shown by these fractions. On the removal of this residual amount of copper ions, the anti-angiogenic activity was lost (see table 2). Addition of copper ions to these binding fractions depleted of copper ions restored their anti-angiogenic activity (see Table 2).

When copper ions were added to an unretained fraction (containing 5 ppm of copper) in an amount that was found to be effective in the binding fraction from the same separation, no increase but rather a decrease in anti-angiogenic activity was observed (see Table 2).

The new fractions of heparin, heparan sulfate and the fractions of products derived from them are characterized by

- that they show enhanced inhibition of angiogenesis in the presence of an angiostatic steroid
- that they have a capacity of binding a certain amount of copper ions strongly
- that they in addition of having a capacity of binding a certain amount of copper ions strongly also must contain enough copper ions to match this capacity in order to show enhanced inhibition of angiogenesis.

The enhanced inhibition of angiogenesis in the presence of an angio-static steroid is show in the egg assay (Table1). The strong binding of copper ions is shown by the high affinity of these fractions for a copper Chelex column, e.g. not being eluted by a water solution, a high salt solution, corresponding to a 0.5 M to 2 M or 3 M solution of NaCl, or a high salt solution of a neutral or almost neutral buffer solution. Strong copper binding is also shown by the difficulty to remove copper ions from these fractions once they have bound the copper ions, e.g. treatment by a copper chelating agent such as a Chelex column in sodium form did not remove the strongly bound copper ions. These were only removed by very extensive treatment with the strong metal chelating agent EDTA. The amount of copper ions necessary for the new fractions to show enhanced inhibition of angiogenesis is determined by the amount of structural elements in these fractions what give rise to the enhanced inhibition of angiogenesis. Copper ions in excess to this amount (e.g.) copper ions which bind to less specific structures) have no additional enhancing effect to the inhibition of angiogenesis of these fractions, but rather a deleterious effect possibly due to a rather high toxicity of copper ions. For a heparin fragment prepared according to Example 1 (e.g. HF-3) an optimal level of copper ions is between 10-500 nmol per μmol binding fraction.

Table 1.

Inhibition of angiogenesis by heparin, heparin fragments and oligosaccharides from heparin fragments in presence of hydrocortison

Example	Product	Amount µg	Percent inhibition[1])	Anti-Xa U/mg
2	Tetrasacch. (TS-1)			
2	Tetrasacch. (TS-2)			
2	Tetrasacch. (TS-3)			
2	Hexasacch. (HS-1	25	24	
2	Hexasacch. (HS-2)	25	24	
2	Hexasacch. (HS-3)	25	48	
1	Heparin fragment (HF-1)	50	33	90
1	Heparin fragment (HF-2)	50	30	
1	Heparin fragment (HF-3)	50	57	16
3	Heparin fragment (HF-4)	12.5	28	
3	Heparin fragment (HF-5)	12.5	15	
3	Heparin fragment (HF-6)	12.5	70	
4	Heparin (H-1)	50	56	138
4	Heparin (H-2)	50	38	
4	Heparin (H-3)	50	72	119
4	Heparin (H-3)	50	17[2])	

1) For details see experimental part

2) Without hydrocortison

Example	Product	Amount µg	Percent inhibition[1])	Anti-Xa U/mg
4	Heparin (H-1)	5	10	138
4	Heparin (H-2)	5	12	
4	Heparin (H-3)	5	36	119
5	Tetrasacch. (TS-4)	25	21	
5	Tetrasacch. (TS-5)	25		
5	Tetrasacch. (TS-6)	25	24	
5	Tetrasacch. (TS-7)	25	48	
6	Hexasacch. HS-4	25	9	
6	Hexasacch. HS-5	25	10	
6	Hexasacch. HS-6	25	12	
6	Hexasacch. HS-7	25	19	
6	Hexasacch. HS-8	25	22	
6	Hexasacch. HS-9	25	57	
7	Decasacch. DS-1	25	26	
7	Decasacch. DS-2	25	13	
7	Decasacch. DS-3	25	54	

1) For details see experimental part

Table 2.

Inhibition of angiogenesis in the presence of hydrocortison by fractions of heparin and heparin fragments according to the invention. The importance of residual copper ions in these fractions.

Compound		µg	nmol	Percent inhibition	Copper ppm	nmole copper / µmole compound
Binding fraction of Heparin fragment (MW 6000)	HF-3	50	8.3	57	255	24
	HF-3A[1])	50	8.3	8	5	0.48
	HF-3B[1])	50	8.3	37	100	9.5
	HF-3C[1])	50	8.3	32	645	61
	HF-3E[1])	50	8.3	53	1100	104
	HF-3D[1])	50	8.3	70	1500	142
Heparin fragment[2]) (MW 8000)	HF-2[1]	50	6.3	30	5	0.64
	HF-2A[1])	50	6.3	8	160	20
Binding fraction of Heparin (MW 13000)	H-3	50	3.8	72	220	46
	H-3	12.5	0.96	42	220	46
	H-3A[1])	12.5	0.96	19	40	8.2
	H-3B[1])	12.5	0.96	38	690	142
	H-3C[1])	12.5	0.96	19	1100	226
Heparin [2])	H-2	50	3.8	38	2	0.4
	H-2A	50	3.8	18	200	42

1) HF-3A was obtained by eliminating strongly bound copper ions from HF-3 (see experimental part, Example 9). HF-3B, HF-3C, HF-3D and HF-3E were obtained by addition of various amounts of cupric

(Cu^{2+})chloride to HF-3A. HF-2A was obtained by addition of the appropriate amount of cupric (Cu^{2+})chloride to HF-2. HF-3A was obtained by eliminating strongly bound copper ions from H-3. H-3B and H-3C was obtained by addition of cupric (Cu^{2+})chloride to H-3A. H-2A was obtained by addition of the appropriate amount of cupric (Cu^{2+})chloride to H-2.

2) Non-binding fraction.

Example 1. Copper binding heparin fragment from heparin fragment obtained by alkaline β-elimination

Sodium heparin from porcine intestinal mucosa (40 g) was dissolved in water (200 ml) and added dropwise while stirring to a solution of Hyamin ^{R} 1622 [Diisobutylphenoxyethoxyethyl]-dimethylbenzylammonium chloride (200 g) in water (1,000 ml). The mixture was stirred for an additional hour and then cooled in refrigerator overnight. The supernatant was separated and the precipitate was washed with water (800 ml) and centrifugated for 1/2 hour. The precipitate was dried at 60°C in vacuum overnight. The sticky gum obtained was dissolved in dichloromethane (750 ml) and α-bromotoluene (85 ml) was added. This solution was stirred for 72 h at 23°C. The benzylester formed was then precipitated by addition of sodium acetate dissolved in methanol (15 w/v, 1,000 ml). The precipitate was centrifugated for 1/2 hour, washed with methanol (500 ml) and centrifugated again. The precipitate obtained was dissolved in water (100 ml) and methanol (25-100 ml). Sodium acetate (15 g) was then added. After stirring for 1/2 h the mixture was filtered through Celite and methanol (1,000 ml) was added to the filtrate. The precipitate was finally washed with methanol, centrifugated and dried in vacuum overnight. Yield of heparin benzylester 26 g.

Heparin benzylester (1.0 g) was dissolved in water (5 ml) and heated to 60°C. Sodium hydroxide (0.40 M, 5 ml) was added and the solution was stirred for 1 1/2 h at 60°C. After cooling the mixture to room temperature, a cation exchange resin (Dowex 50 W-X8 H) was added to neutralize the basic solution. The resin was filtered off and washed with water (1 ml). The pH of the combined solutions (filtrate and washings) was adjusted to pH 7 by addition of dilute sodium hydroxide and then freeze-dried. Yield 1.0 g. The average molecular weight of this heparin fragment (HF-1) was 8,000 as determined by gel filtration on Sephadex G-75, using heparin fragments of known degree of polymerization as references. Anticoagulant activity was measured by an anti-Xa assay utilizing the chromogenic substrate S-2222 (Kabi Diagnostica Stockholm, Sweden), according to Andersson et al., Thromb. Res. 1979, 15, 531. The anti-Xa activity of this heparin fragment (HF-1) was 90 U/mg.

A copper chelate affinity chromatography column was prepared in the following way. Chelex 100 (Bio-Rad) 50 ml in sodium form was added to a solution of cupric (Cu^{2+}) chloride (500 ml, 0.5 M). The mixture was left for 24 h and then filtered. The affinity gel was washed with distilled water (3 x 100 ml). The affinity gel was poured into distilled water and deaerated. A column was prepared by pouring Chelex 100 containing sodium ions (5 ml) into a chromatographic column (25 x 1.6 cm) and on top of this gel the copper Chelex gel. The column was washed with ammonia (100 ml, 0.5 M) and water (250 ml). Heparin fragment (HF-1) (5 g), obtained from heparin by alkaline β-elimination as described above was dissolved in water (20 ml) and applied to the top of the copper Chelex column. Water was used as an eluent at 1.2 cm/h and when no more heparin fragment was eluted as detected by a UV-monitor fixed at 240 nm, the eluent was changed to aqueous ammonia (2 M). The solvent was evaporated under vacuum from the fraction eluted by water and the fraction eluted with ammonia. They were then dissolved in distilled water and treated with Chelex 100 containing sodium ions, filtered and freeze-dried. From the water solution, a fraction (3.2 g) of the original heparin fragment was obtained. This fraction, designated Heparin fragment HF-2, had very low, or no affinity for the copper Chelex column. The fraction that eluted with aqueous ammonia was dissolved in distilled water and subjected to ultrafiltration on a Diaflo membrane 5 YCO5 in an Amicon 8050 ultrafilter cell. The retentate was then freeze-dried. The freeze-dried product was dissolveld in sodium chloride (2 M) and again subjected to ultrafiltration on the same filter, washed thoroughly with water until free from chloride ions and then freeze-dried to give the fraction of the original heparin fragment that had

been retained on the copper Chelex column 0.2 g. This fraction of the heparin fragment HF-1 was designated heparin fragment HF-3. The retained fraction HF-3 had an average molecular w ight of 6,000 as determined by gel filtration on Sephadex G-75. By elemental analysis N was 2.4% and sulfur 9.5%, and Cu 255 ppm. Uronic acids were 30 w/w% as determined by the carbazol sulfuric acid method according to Bitter and Muir, Anal. Biochem. 1962, 4, 330.

The anti-Xa activity of this heparin fragment (HF-3) was 16 U/mg. Fraction HF-3 did only contain glucosamine and no galactosamine as determined by amino acid analysis. A ^{1}H-NMR spectra in D_2O/NaOD did not show any signal at 2.7 ppm due to H-2 of glucosamine having a free amino group in that position.

Example 2. Copper binding oligosaccharides obtained from oligosaccharides which were obtained from a heparin fragment produced by alkaline β-elimination depolymerization of sodium heparin

Sodium heparin from porcine intestinal mucosa (40 g) was dissolved in water (200 ml) and added dropwise while stirring to a solution of Hyamine^R 1622 [(diisobutylphenoxyethoxyethyl)-dimethylbenzylammonium chloride] (200 g) in water (1,000 ml). The mixture was stirred for an additional hour and then centrifugated. The supernatant was separated and the precipitate was washed with water (800 ml) and centrifugated for 1/2 hour. The precipitate was dried at 60 °C in vacuum overnight. The sticky gum obtained was dissolved in dichloromethane (750 ml) and methyl iodide (45 ml) was added. This solution was stirred for 72 h at 23 °C. The methylester formed was then precipitated by addition of sodium acetate dissolved in methanol (15% w/w, 1,000 ml). The precipitate obtained was dissolved in water (100 ml) and methanol (25-100 ml). Sodium acetate (15 g) was then added. After stirring for 1/2 h the mixture was filtered through Celite and methanol (1,000 ml) was added to the filtrate. The precipitate was centrifugated and the supernatant decanted. This procedure was repeated until a precipitate was obtained, which was completely water soluble. The precipitate was finally washed with methanol, centrifugated and dried in vacuum overnight. Yield of heparin methylester 23 g.

Heparin methylester (1.0 g) was dissolved in water (5 ml) and heated to 60 °C. Sodium hydroxide (0.40 M, 5 ml) was added and the solution was stirred for 1 1/2 h at 60 °C. After cooling the mixture to room temperature, a cation exchange resin (Dowex 50 W-X8 H) was added to neutralize the basic solution. The resin was filtered off and washed with water (1 ml). The pH of the combined solutions was adjusted to pH 7 by addition of dilute sodium hydroxide and then freeze-dried. Yield 0.98 g. The average molecular weight of this heparin fragment was 3,500.

This heparin fragment (MW 3,500) (1 g) was dissolved in sodium chloride (0.25 M, 5 ml) and applied to a gel permeation column (5 x 180 cm P-6, Bio-Rad). The column was eluted by sodium chloride (0.25 M) at 5.8 cm/h, using UV-detection at 214 nm. Fractions were collected and desalted on Sephadex G-10 using water at 3.6 cm/h as eluent and detection by refractive index measurement. After freeze-drying, the following fractions were obtained:

Disaccharides	5 mg
Tetrasaccharides	36 mg
Hexasaccharides	65 mg
Octasaccharides	89 mg
Decasaccharides	118 mg
Dodecasaccharides	95 mg
Tetradecasaccharides	71 mg
Hexadecasaccharides	83 mg
Octadecasaccharides and higher saccharides	305 mg

Tetrasaccharides (TS-1, 450 mg) obtained as described above were dissolved in water (5 ml) and applied to a copper Chelex column, prepared as described in Example 1. Elution was carried out at 1.2 cm/h with water as eluent and monitored with UV-detection at 240 nm. When the unretained (non binding) tetrasaccharides had been eluted, the eluant was changed to aqueous ammonia (2 M) that yielded a fraction of tetrasaccharides that had been retained by (bound to) the column. These two fractions were evaporated. The binding fraction was evaporated repeatedly. After evaporation of solvent the fractions were dissolved in distilled water and treated with Chelex containing sodium ions, filtered and purified by chromatography on Sephadex G-10 as described for the isolation of the tetrasaccharides above. After freeze-drying, a tetrasaccharide fraction was obtained from the elution with water, which was very little, or not at all retained on the copper Chelex column. Yield 435 mg (TS-2). The elution with ammonia gave another fraction. This

fraction contained tetrasaccharides retained by (bound to) the copper Chelex column. Yield 6.5 mg (TS-3).

In th same way hexasaccharides (HS-1, 550 mg) obtained as described above, from heparin fragment obtained by alkaline β-elimination of heparin, were fractionated on the copper Chelex column as described above to give a hexasaccharide fraction that was very little, or not at all retained. Yield 526 mg (HS-2). Also a h xasaccharide fraction was obtained, yield 8.9 mg (HS-3) which had been retained on the copper Chelex column. Neither the retained tetrasaccharide (TS-3), nor the retained hexasaccharide (HS-3) showed any ^{1}H-NMR signal in D_2O/NaOD at 2.7 ppm due to H-2 of glucosamine having a free amino group at that position.

Each one of the larger oligosaccharides was fractionated in the same way as for the tetra- and hexasaccharides except that desalting was performed on an ultrafilter membrane Diaflo 5YCO5 (Amicon Corp.). Yields of the fractions which were retained on the copper Chelex column were 0.3-3%.

Example 3. Copper binding heparin fragment from heparin fragment obtained by partial depolymerization of sodium heparin by nitrous acid

Sodium heparin from porcine intestinal mucosa was partially depolymerized at pH 1.5 by nitrous acid formed in situ by addition of a solution of sodium nitrite (5% w/w). Anhydromannose groups were then reduced at 3°C by an excess of sodium borohydride. After destruction of excess sodium borohydride by acetic acid and neutralizing the solution by addition of dilute sodium hydroxide, a portion of the solution was subjected to gel permeation chromatography on Sephadex G-15, with water as an eluent. A heparin fragment (Heparin fragment HF-4), containing tetradeca to hexadecasaccharides as the main components according to HPLC gel-permeation chromatography, was collected. HPLC gel permeation chromatography was done on two TSK G 3,000 SW columns, each 600 mm x 7.5 mm i.d., connected in series. A TSK SWP column (75 mm x 7.5 mm i.d.) was used as a guard column. Mobilephase was 0.2 M sodium acetate. Flowrate was 0.5 ml/min. Peaks were detected by refractive index measurements using heparin oligosaccharides of known sizes as reference.

A copper chelate affinity chromatography column was prepared as follows: Chelex 100 (Bio-Rad, 50 ml, 100-200 mesh) containing sodium ions was washed with water and packed in a column (2.6 x 30 cm). The Chelex gel was saturated with copper ions by pumping a solution of cupric (Cu^{2+}) chloride (0.5 M; 500 ml) through the column at 3.8 cm/h. Excess cupric chloride was washed away with distilled water. The column was then equilibrated with sodium chloride (0.5 M), containing sodium phosphate (0.02 M). After the copper Chelex column, a column containing Chelex with sodium ions was placed. To this two column system, heparin fragment HF-4, obtained as described above (500 mg), dissolved in sodium chloride, sodium phosphate (0.5 M, 0.02 M) was applied. Heparin fragment which was only very little, or not at all retained on the copper chelex column was eluted with sodium chloride, sodium phosphate (0.5 M, 0.02 M). Elution was continued until no more fragment could be detected by UV at 214 nm.

This fraction of heparin fragment HF-4 was called heparin fragment HF-5. After desalting on Sephadex G-15 and freeze-drying, the yield was 395 mg. The main peaks of this fraction on HPLC were tetradeca to hexadecasaccharides. Another fraction of heparin fragment which was retained by (bound to) the copper Chelex column was eluted by ammonium chloride (2 M) containing sodium chloride (0.5 M). After chromatography on Sephadex G-15, a yield of 0.7 mg was obtained. This fraction of heparin fragment was called heparin fragment HF-6. According to HPLC it consisted mainly of octa- to hexadecasaccharides.

Example 4. Copper binding heparin fraction from standard sodium heparin

A copper Chelex column (5 x 25 cm) containing about 500 ml of copper ion containing gel was prepared according to Example 3. After that column, a sodium Chelex column (1.6 x 10 cm) was placed. To this two column system, a standard sodium heparin USP (20 g, anti-Xa 138 U/mg, H-1) dissolved in sodium chloride (0.5 M) was added. Heparin that was only little, or not at all retained by the copper ions on the gel, was eluted with sodium chloride (0.5 M, 1.8 l). When a stable baseline was obtained (UV-detection at 214 nm), elution was continued using aqueous ammonia (2 M) containing sodium chloride (0.5 M).

A portion of the heparin fraction that was not at all, or very little retained was treated with Chelex containing sodium ions and then freeze-dried (heparin fraction H-2). The retained heparin fraction was evaporated, desalted on Sephadex G-15, freeze-dried and treated with Chelex containing sodium ions and then desalted on Sephadex G-15 using distilled water. After freeze-drying, a heparin fraction (heparin fraction H-3) was obtained that had been retained on the copper Chelex column (88 mg). Anti-Xa activity was 119 U/mg. By elemental analysis N was 2.1%, S was 10.7%, Na was 10.3%, and Cu 220 ppm. The only amino sugar in this fraction according to amino acid analysis was glucosamine. Uronic acids were 28±3% w/w as determined by the carbazol sulfuric acid method according to Bitter and Muir, Anal.

Biochem. 4, (1962) 330. The uronic acid content of the standard sodium heparin used as starting material was 30±3% w/w.

Example 5. Preparation of a copper-binding tetrasaccharide fraction from heparin fragment obtained by partial depolymerization of sodium heparin by nitrous acid

Depolymerized heparin from Example 3 was precipitated by adding 8 volumes of ethanol. 5 g of the product thus obtained was dissolved in water, 12 ml, and subjected to gel permeation chromatography on a Biogel P-6 gel, 180 x 5 cm, with 0.25 M sodium chloride as an eluent at 3.7 cm/h. Chromatography was monitored by a refractive index detector and fractions were pooled to obtain size homogenous oligosaccharides. The respective fractions were concentrated. The smaller size fractions were desalted by chromatography on a Sephadex G-10 column (85 x 5 cm) with water at 3.6 cm/h as an elutent. Detection of carbohydrate containing material was performed by a refactive index detector. Larger sized oligosaccharides (≥10 monosaccharide units) were desalted by ultrafiltration in a Amicon 8400 cell with a YCO5 filter. The yields were as follows:

Tetrasaccharides	0.51 g
Hexasaccharides	0.71 g
Octasaccharides	0.35 g
Decasaccharides	0.51 g
Dodecasaccharides	0.17 g
Tetradecasaccharides	0.31 g
Hexadecasaccharides	0.15 g
Octadecasaccharides	0.14 g
Eicosasaccharides and larger saccharides	0.43 g

The tetrasaccharide fraction (6.0 g) (TS-4) was dissolved in water (10 ml) and applied to a Chelex 100 column (25 x 1.6 cm), copper form. Water at 1.2 cm/h was used as an eluent to obtain a fraction with low affinity for copper. Detection was performed with a ultraviolet UV 214 detector. When the first fraction was eluted, the eluent was changed to ammonia (1,000 ml, 2 M) and a second fraction was collected. The respective fractions were concentrated and then treated with Chelex 100, sodium form. Finally the fractions were chromatographied on Sephadex G-10 with water at 3.6 cm/h as an eluent to give the nonbinding fraction (5.8 g) (TS-5) and binding fraction (84 mg).

The binding fraction was dissolved in 0.25 M sodium chloride and applied to gel permeation chromatography on a Biogel P-6 gel, 180 x 5 cm column and eluted with 0.25 M sodium chloride as above. The tetrasaccharide fraction was collected and desalted on Sephadex G-10 with water as an eluent to give, after freeze-drying, 70 mg product with a copper content of 3,900 ppm (TS-6). The binding fraction (18 mg) was dissolved in water (5 ml) and sodium chloride (20 mg) and Chelex 100 was added. The mixture was left for two hours and then filtered. After concentration the remainder was desalted on a G-10 column as above. The tetrasaccharide was collected and freeze-dried to give 9.5 mg product with a copper content of 2,900 ppm (TS-7). The tetrasaccharidic nature of TS-7 was confirmed by HPLC gel permeation chromatography according to example 3.

Example 6

A hexasaccharide fraction (HS-4) prepared according to example 5 (1.15 g) was dissolved in 0.5 M sodium chloride and applied to a two column system consisting of a copper Chelex column followed by a sodium Chelex column. The columns were eluted by a stepwise increasing concentration of ammonium chloride. Each fraction was desalted by repeated chromatography on Sephadex G-15 then treated with a large excess sodium chloride and desalted again yielded the following fractions. Fractions HS-5 and HS-9 were further purified by treatment with sodium Chelex.

Fraction no	Eluent	Volume (ml)	Yield (mg)	Cu-content (ppm)
HS-5	0.5 M NaCl	270	897	18
HS-6	0.5 M NaCl 0.7 M NH_4Cl	500	75	550
HS-7	0.5 M NaCl 1.4 M NH_4Cl	340	10	120
HS-8	0.5 M NaCl 2.0 M NH_4Cl	780	32	640
HS-9	0.5 M NaCl 3.0 M NH_4Cl	700	4	425

Fractions HS-9 was confirmed by HPLC gel-permeation chromatography to be a hexasaccharide. A1H-NMR spectrum is shown in Figure 1.

Example 7

Chelating Sepharose 6B (Pharmacia; 110 ml) was placed in a beaker. Cupric (Cu^{2+}) sulfate (0.5 M, IL) was added and the gel was left over night. The copperion containing gel was filtered on a sintered glassfunnel and thoroughly washed with water (10 l). It was than added to a sodium chloride solution (1M, 300 mL) containing sodium phosphate (0.1 M; pH 7.5), (buffer A). The gel was deaerated and transferred to a chromatography column (40 x 2.6 cm) and washed with buffer A (100 mL). A decasaccharide (DS-1) obtained according to example 5 (5 g) was dissolved in buffer A (15 mL) and placed on the top of the copperion containing column. Elution was performed with buffer A at 6.8 cm/h. The eluate was monitored with a fixed wavelength UV detector (UV-214, Pharmacia), and fractions were collected (20 min/tube). After 30 min a non-binding material emerged. Fractions containing the non-binding material was pooled and concentrated, desalted and freeze-dried to give decasaccharide DS-2. The copper column was then washed with sodium chloride (3 M) containing sodium phosphate (0.1 M, pH 7.5) buffer B at 6.8 cm/h for 7 h. No peaks appeared in the chromatogram during this period. The eluent was then changed to ammonium chloride (2 M) and chromatography was continued until a peak emerged. Fractions corresponding to this peak were collected and pooled, concentrated and desalted in an Amicon 8000 ultrafilter cell using a YCO 5 membrane. The retentate was washed with sodium chloride (1 M, 2 x 300 mL) and then with water until free from chloride ions. The retentate was freeze-dried to give a binding fraction (DS-3), 14 mg, Cu content 640 ppm. No further peaks appeared on continued chromatography with 2 M ammonium chloride over night.

Biological Tests

The angiogenic response of the chorioallantoic membrane (CAM) to heparin, heparan sulfate or products containing heparin and/or heparan sulfate constituents were investigated on fertilized eggs. Non-incubated (0-day) fertilized eggs obtained from Linköpings Kontrollhönseri, Linköping, Sweden were stored in a low temperature incubator 13-17 °C until ready for incuation at 37 °C in an Egg Incubator (Andersson & Bonde TYP 40). Incubated eggs (37 °C) are cracked on the 3rd day of incubation (3-day) and the entire

content is poured into a Petri dish. Only eggs with unbroken yolk were used for further incubation in a CO_2 tissue culture incubator with 3% CO_2 at 37°C with elevated relative humidity. After 3 days incubation (6-day) in CO_2 atmosphere the eggs are implanted with a methy cellulose dish prepared as described below. Methyl cellulose is added to triple distilled water at a conc ntration of 0.5%, then auto-claved 30 minutes at 138 kPa and 120°C for st rility. This mixture will contain a ball of gelled methyl cellulose. Subsequent slow agitation for 2-3 days at 5°C insures complete dissolving of solution. The solution is then kept in a refrigerator until ready for use. The test material is suspended in the methyl cellulose solution at room temperature to a final concentration of 5 to 50 µg/10 µl. Aliquots of 10 µl methylcellulose containing the test sample, are deposited onto the end of a teflon rod (3 mm diam.). When the methyl cellulose test material disc is dry (30-40 min) it is lifted off the teflon with 2 fine foreceps and placed on the peripheral part of a 6-day CAM in order to avoid non-growing vessels. On day 8 the growing vessels under and around the transparent disc are examined under a dissecting microscope (x 26 to x 40 magnification). The inhibition of vessel growth is scored either as no inhibition of vessel growth or as inhibiting vessel growth. Each compound is tested at a minimum of 15 eggs and the scoring is performed by two technicians who are uninformed concerning the nature and concentration of the test substance.

The results are expressed as per cent eggs showing inhibition.

Example 9. Copper content of fractions of heparin fragments of the invention (Table 2)

Heparin fragments HF-1, HF-2 and HF-3 were analyzed for copper by atomic absorption spectroscopy. They were found to contain 9 ppm, 5 ppm and 255 ppm, respectively. Further treatment of a solution of heparin fragment HF-3 in distilled water with Chelex 100 containing sodium ions did not give any decrease in the copper content. In order to reduce the copper content below 10 ppm, the following steps were employed. For all the steps carried out, a Diaflo membrane 5YCO5 in an Amicon 8050 ultrafilter cell was used.

Heparin fragment (HF-3) was dissolved in sodium chloride (2 M, 50 ml) and washed by additional sodium chloride (2 M; 250 ml) and then by distilled water (200 ml) and then freeze-dried. This freeze-dried fragment was then dissolved in ethylendiaminetetraacetic acid (EDTA; 0.1 M; pH 7.0; 50 ml) and washed with an additional 250 ml of the same solution. It was then successively washed with sodium chloride (1 M, 250 ml) and distilled water (250 ml) and then freeze-dried. This freeze-dried fragment was once more treated in the same way using twice the washing volumes and then freeze-dried. The heparin fragment called HF-3A, now had a copper content of 5 ppm. All the freeze-dried intermediates above were analyzed for copper but only after all steps the copper content was below 10 ppm.

For addition of copper ions to heparin fragment HF-3A a stock solution of cupric(Cu^{2+})chloride was prepared as follows: Cupric(Cu^{2+})chloride x $2H_2O$ (27.1 mg) was weighed into a graduate flask and triple distilled water was added to the mark, 10 ml. From this solution 1 ml was drawn into a second graduate flask and triple distilled water was added to the mark, 1,000 ml. This solution contained 1.01 µg Cu/ml H_2O. 5.2 mg of heparin fragment containing 5 ppm of copper (HF-3A) was dissolved in triple distilled water (2.0 ml). 0.51 ml of the stock solution was added and the mixture was left at room temperature for 4 hours and then freeze-dried to give heparin fragment HF-3B. The copper content by atomic absorption analysis was 100 ppm.

Heparin fragment HF-3A (5.9 mg) was dissolved in triple distilled water (2.0 ml) and 3.80 ml of the stock solution was added and the mixture was left at room temperature for 4 hours and then freeze-dried to give heparin fragment HF-3C, the copper content by atomic absorption analysis was 645 ppm.

Heparin fragment HF-3A (4.6 mg) was dissolved in 2.0 ml of triple distilled water and 6.90 ml of the stock solution was added and the mixture was left for 4 hours at room temperature and then freeze-dried to give heparin fragment HF-3D. The copper content by atomic absorption was 1,500 ppm.

HF-3C (3.6 mg) and HF-3D (4.1 mg) was dissolved in water (10 ml). The solution was then freeze-dried to give heparin fragment HF-3E (7.6 mg) containing 1100 ppm Cu as determined by atomic absorption;

For addition of copper ions to heparin fragment HF-2, 20.0 mg of HF-2 was dissolved in 2.0 ml triple distilled water and 3.17 ml of the stock solution was added and the mixture was left at room temperature for 4 hours and then freeze-dried to give heparin fragment HF-2A. The copper content by atomic absorption spectroscopy was 160 ppm.

The fractions of heparin were treated in the same way as the fractions of the heparin fragments in order to give the heparin fractions H-3A, H-3B, H-3C and H-2A.

It is seen in Table 2 that the anti-angiogenic effect is almost totally lost when the copper content is reduced. For example, the percent inhibition is 8 for fragment HF-3A compared with 57 for fragment HF-3. Addition of copper ions restored the activity, as is seen e.g. in fragments HF-3B, HF-3D, and HF-3E

exhibiting 37, 70, and 53% inhibition, respectively. A similar effect as for heparin fragments is seen also for Heparin, compare H-3A and H-3B with H-3 in Table 2.

Claims

Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Medicament for the treatment of an ailment where reduced angiogenesis is desired, comprising a complex of
   (a) a metal ion which is copper and
   (b) a fraction of heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, heparin fragments, heparan sulfate fragments and oligosaccharides derived from heparin or from heparan sulfate, or a salt of such fractions, said fraction having a molecular weights from 500 to 35.000 and said salt being a physiologically acceptable salts such as sodium, calcium or ammonium salts,
   which fractions bind to the said metal ion and
   said complex containing from 5 - 1,000 nmole metal per µmole of component (b).

2. A medicament according to claim 1 comprising a complex wherein the amount of copper is from 10 to 1.000 nmole per µmole of component (b).

3. A medicament according to claims 1 or 2 comprising a complex wherein component (b) is heparin or a low molecular weight heparin or a heparin fragment or an oligosaccharide derived from heparin.

4. A medicament according to claim 3 comprising a complex wherein the oligosaccharide is a tetrasaccharide, a hexasaccharide, an octasaccharide, a decasaccharide, a dodecasaccharide, a tetradesaccharide or a hexadecasaccharide.

5. A medicament according to claims 1 or 2 comprising a complex wherein component (b) is heparan sulfate, low molecular weight heparan sulfate or heparan sulfate fragments or an oligosaccharides derived from heparan sulfate.

6. A medicament according to claim 5 comprising a complex wherein said oligosaccharide is a tetrasaccharide, a hexasaccharide, an octasaccharide, a decasaccharide, a dodecasaccharide, a tetradesaccharide or a hexadecasaccharide.

7. A medicament according to any of claims 1 to 6 comprising a complex wherein the component (b) is in the form of a salt.

8. A medicament according to claim 7 comprising a complex wherein the salt is a sodium or a calcium salt.

9. A medicament according to claim 8 comprising a complex wherein the salt is a sodium salt.

10. A process for the preparation of a complex according to claims 1-9 consisting of adding heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, heparin fragments, a heparan sulfate fragment and oligosaccharide derived from heparin or from heparan sulfate to a solid matrix containing iminodiacetic acid groups to which a copper ion is bound, separating the fraction of added material that does not bind to the copper ions on the matrix and thereafter desorption of the fraction bound to the the matrix.

11. Use of a complex of
   (a) a metal ion selected from copper, calcium, manganese, iron and zinc ions and
   (b) a fraction of heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, heparin fragments, heparan sulfate fragments and oligosacarides derived from heparin or from heparan sulfate, or a salt of such fractions, said fraction having a molecular weights from 500 to 35.000 and said salt being a physiologically acceptable salts such as sodium, calcium or ammonium salts,
   which fraction bind to the said metal ion and

said complex containing from 5 to 1.000 nmole metal per µmole of component (b)
in the preparation of a medicament for the treatment of an ailment, where reduced angiogenesis is desired.

12. Use according to claim 11 in which the medicament comprises the complex in conjunction with an angiostatic component, especially a so-called angiostatic steroid component.

Claims for the following Contracting States : ES, GR

1. A process for the preparation of a medicament for the treatment of an ailment where reduced angiogenesis is desired, in which the medicament comprises a complex of
(a) a metal ion which is copper and
(b) a fraction of heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, heparin fragments, heparan sulfate fragments and oligosacarides derived from heparin or from heparan sulfate, or a salt of such fractions, said fraction having a molecular weights from 500 to 35.000 and said salt being a physiologically acceptable salts such as sodium, calcium or ammonium salts,
which fraction bind to the said metal ion and
said complex containing from 5 - 1,000 nmole metal per µmole of component (b)
and in which the process in characterized by adding fraction (b) to a solid matrix containing iminodiacetic acid groups to which a copper ion is bound, separating the fraction of added material that does not bind to the copper ions on the matrix and thereafter desorption of the fraction bound to the the matrix.

2. A process according to claim 1 in which the medicament comprises a complex wherein the amount of copper is from 10 to 1.000 nmole per µmole of component (b).

3. A process according to claims 1 or 2 in which the medicament comprises a complex wherein component (b) is heparin or a low molecular weight heparin or a heparin fragment or an oligosaccaride derived from heparin.

4. A process according to claim 3 in which the medicament comprises a complex wherein the oligosaccaride is a tetrasaccaride, a hexasaccaride, an octasaccaride, a decasaccaride, a dodecasaccaride, a tetradesaccaride or a hexadecasaccaride.

5. A process according to claims 1 or 2 in which the medicament comprises a complex wherein component (b) is heparan sulfate, low molecular weight heparan sulfate or heparan sulfate fragments or an oligosacarides derived from heparan sulfate.

6. A process according to claim 5 in which the medicament comprises a complex wherein said oligosaccaride is a tetrasaccaride, a hexasaccaride, an octasacaride, a decasaccaride, a dodecasacaride, a tetradesaccaride or a hexadecasaccaride.

7. A process according to any of claims 1 to 6 in which the medicament comprises a complex wherein the component (b) is in the form of a salt.

8. A process according to claim 7 in which the medicament comprises a complex wherein the salt is a sodium or a calcium salt.

9. A process according to claim 8 in which the medicament comprises a complex wherein the salt is a sodium salt.

10. Use of a complex of
(a) a metal ion selected from copper, calcium, manganese, iron and zinc ions and
(b) a fraction of heparin, heparan sulfate, low molecular weight heparin, low molecular weight heparan sulfate, heparin fragments, heparan sulfate fragments and oligosacarides derived from heparin or from heparan sulfate, or a salt of such fractions, said fraction having a molecular weights from 500 to 35.000 and said salt being a physiologically acceptable salts such as sodium, calcium or

ammonium salts,
which fraction bind to the said metal ion and
said complex containing from 5 to 1.000 nmole metal per µmole of component (b)
in the preparation of a medicament for the treatment of an ailment, where reduced angiogenesis is desired.

11. Use according to claim 10 in which the medicament comprises the complex in conjunction with an angiostatic component, especially a so-called angiostatic steroid component.

Patentansprüche
Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Heilmittel zur Behandlung einer Unpässlichkeit wo eine verminderte Angiogenesis erwünscht ist, umfassend einen Komplex von
(a) einem Metallion, welches Kupfer ist, und
(b) einem Anteil von Heparin, Heparansulfat, Heparin geringer Molekülmasse, Heparansulfat geringer Molekülmasse, Heparinfragmente, Heparansulfatfragmente und Oligosaccharide, abgeleitet von Heparin oder von Heparansulfat, oder einem Salz solcher Anteile, wobei diese Anteile Molekulargewichte von 500 bis 35.000 haben und das Salz ein physiologisch annehmbares Salz wie Natrium, Kalzium oder Ammoniumsalz ist, und wo die Anteile an diesem Metallion gebunden sind und dieser Komplex 5-1.000 nMol Metall pro µMol der Komponente (b) enthält.

2. Heilmittel gemäss Anspruch 1, umfassend einen Komplex, worin die Menge Kupfer 10 bis 1.000 nMol pro µMol der Komponente (b) beträgt.

3. Heilmittel gemäss der Ansprüche 1 oder 2, umfassend einen Komplex, worin die Komponente (b) Heparin oder Heparin geringer Molekülmasse oder ein Heparinfragment oder ein Oligosaccharid abgeleitet von Heparin ist.

4. Heilmittel gemäss Anspruch 3, umfassend einen Komplex, worin das Oligosaccharid ein Tetrasaccharid, ein Hexasaccharid, ein Octasaccharid, ein Decasaccharid, ein Dodecasaccharid, ein Tetradesaccharid oder ein Hexadecasaccharid ist.

5. Heilmittel gemäss der Ansprüche 1 oder 2, umfassend einen Komplex, worin die Komponente (b) Heparansulfat, Heparansulfat geringer Molekülmasse oder Heparansulfatfragmente oder ein Oligosaccharid, abgeleitet von Heparansulfat, ist.

6. Heilmittel gemäss Anspruch 5, umfassend einen Komplex, worin das Oligosaccharid ein Tetrasaccharid, ein Hexasaccharid, ein Octasaccharid, ein Decasaccharid, ein Dodecasaccharid, ein Tetradesaccharid oder ein Hexadecasaccharid ist.

7. Heilmittel nach einem der Ansprüche 1 bis 6, umfassend einen Komplex, worin die Komponente (b) in Salzform vorliegt.

8. Heilmittel gemäss Anspruch 7, umfassend einen Komplex worin das Salz ein Natrium- oder Kalziumsalz ist.

9. Heilmittel gemäss Anspruch 8, umfassend einen Komplex worin das Salz ein Natriumsalz ist.

10. Verfahren zur Herstellung eines Komplexes gemäss den Ansprüchen 1-9, bestehend aus zufügen von Heparin, Heparansulfat, Heparin geringer Molekülmasse, Heparansulfat geringer Molkülmasse, Heparinfragmente, ein Heparansulfatfragment und Oligosaccharide abgeleitet von Heparin oder von Heparansulfat, zu einer festen Matrix, enthaltend Iminodiessigsäuregruppen an welche ein Kupferion gebunden ist, abtrennen des Anteiles von zugefügtem Material, welches nicht an die Kupferionen auf der Matrix bindet und anschliessender Desorption des Anteiles der an die Matrix gebunden ist.

11. Verwendung eines Komplexes aus
(a) einem Metallion ausgewählt von Kupfer, Kalzium, Mangan, Eisen und Zinkionen, und

(b) einem Anteil von Heparin, Heparansulfat, Heparin geringer Molekülmasse, Heparansulfat geringer Molekülmasse, Heparinfragmente, Heparansulfatfragmente und Oligosaccharide abgeleitet von Heparin oder von Heparansulfat oder einem Salz solcher Anteile, wobei diese Anteile Molekulargewichte von 500 bis 35.000 haben und das Salz ein physiologisch annehmbares Salz wie Natrium, Kalzium oder Ammoniumsalz ist, und wo die Anteile an diesem Metallion gebunden sind und dieser Komplex 5 bis 1.000 nMol Metall pro µMol der Komponente (b) enthält, zur Herstellung eines Heilmittels zur Behandlung einer Unpässlichkeit, wo eine verminderte Angiogenesis erwünscht ist.

12. Verwendung gemäss Anspruch 11, worin das Heilmittel den Komplex in Verbindung mit einer angiostatischen Komponente, besonders einer sogenannten angiostatischen Steroid-Komponente, umfasst.

Patentansprüche für folgende Vertragsstaaten : ES, GR

1. Verfahren zur Herstellung eines Heilmittels zur Behandlung einer Unpässlichkeit wo eine verminderte Angiogenesis erwünscht ist, wobei das Heilmittel einen Komplex umfasst, aus
(a) einem Metallion, welches Kupfer ist, und
(b) einem Anteil von Heparin, Heparansulfat, Heparin geringer Molekülmasse, Heparansulfat geringer Molekülmasse, Heparinfragmente, Heparansulfatfragmente und Oligosaccharide, abgeleitet von Heparin oder von Heparansulfat, oder einem Salz solcher Anteile, wobei diese Anteile Molekulargewichte von 500 bis 35.000 haben und das Salz ein physiologisch annehmbares Salz wie Natrium, Kalzium oder Ammoniumsalz ist, und wo die Anteile an diesem Metallion gebunden sind und dieser Komplex 5-1.000 nMol Metall pro µMol der Komponente (b) enthält, dadurch gekennzeichnet, dass der Anteil (b) einer festen Matrix enthaltend Iminodiessigsäuregruppen an welche ein Kupferion gebunden ist, zugegeben wird, abtrennen des Anteils von zugefügtem Material, welches nicht an die Kupferionen auf der Matrix bindet und anschliessender Desorption des Anteils der an die Matrix gebunden ist.

2. Verfahren gemäss Anspruch 1, worin das Heilmittel einen Komplex umfasst, worin die Menge Kupfer 10 bis 1.000 nMol pro µMol der Komponente (b) beträgt.

3. Verfahren gemäss den Ansprüchen 1 oder 2, worin das Heilmittel einen Komplex umfasst, worin die Komponente (b) Heparin oder Heparin geringer Molekülmasse oder ein Heparinfragment oder ein Oligosaccharid abgeleitet von Heparin, ist.

4. Verfahren gemäss Anspruch 3, worin das Heilmittel einen Komplex umfasst, worin das Oligosaccharid ein Tetrasaccharid, ein Hexasaccharid, ein Octasaccharid, ein Decasaccharid, ein Dodecasaccharid, ein Tetradesaccharid oder ein Hexadecasaccharid ist.

5. Verfahren gemäss den Ansprüchen 1 oder 2, worin das Heilmittel einen Komplex umfasst, worin die Komponente (b) Heparansulfat, Heparansulfat geringer Molekülmasse oder Heparansulfatfragmente oder ein Oligosaccharid, abgeleitet von Heparansulfat, ist.

6. Verfahren gemäss Anspruch 5, worin das Heilmittel einen Komplex umfasst, worin das Oligosaccharid, ein Tetrasaccharid, ein Hexasaccharid, ein Octasaccharid, ein Decasaccharid, ein Dodecasaccharid, ein Tetradesaccharid oder ein Hexadecasaccharid, ist.

7. Verfahren nach einem der Ansprüche 1 bis 6, worin das Heilmittel einen Komplex umfasst, worin die Komponente (b) in Salzform vorliegt.

8. Verfahren gemäss Anspruch 7, worin das Heilmittel einen Komplex umfasst, worin das Salz ein Natrium- oder Kalziumsalz ist.

9. Verfahren gemäss Anspruch 8, worin das Heilmittel einen Komplex umfasst, worin das Salz ein Natriumsalz ist.

10. Verwendung ines Komplexes aus
(a) einem Metallion ausgewählt von Kupfer, Kalzium, Mangan, Eisen und Zinkionen, und

(b) einem Anteil von Heparin, Heparansulfat, Heparin geringer Molekülmasse, Heparansulfat geringer Molekülmasse, Heparinfragmente, Heparansulfatfragmente und Oligosaccharide abgeleitet von Heparin oder von Heparansulfat oder einem Salz solcher Anteile, wobei diese Anteile Molekulargewichte von 500 bis 35.000 haben und das Salz ein physiologisch annehmbares Salz wie Natrium, Kalzium oder Ammoniumsalz ist, und wo die Anteile an diesem Metallion gebunden sind und dieser Komplex 5 bis 1.000 nMol Metall pro µMol der Komponente (b) enthält, zur Herstellung eines Heilmittels zur Behandlung einer Unpässlichkeit, wo eine verminderte Angiogenesis erwünscht ist.

11. Verwendung gemäss Anspruch 10, worin das Heilmittel den Komplex in Verbindung mit einer angiostatischen Komponente, besonders einer sogenannten angiostatischen Steroid-Komponente, umfasst.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Médicament pour le traitement d'une maladie dans laquelle on désire réduire l'angiogenèse, comprenant un complexe de
   (a) un ion métallique qui est le cuivre et
   (b) une fraction d'héparine, d'héparane-sulfate, d'héparine de bas poids moléculaire, d'héparane-sulfate de bas poids moléculaire, de fragments d'héparine, de fragments d'héparane-sulfate et d'oligosaccharides dérivés de l'héparine ou de l'héparane-sulfate, ou un sel de ces fractions, lesdites fractions ayant des poids moléculaires de 500 à 35 000 et lesdits sels étant des sels physiologiquement acceptables comme les sels de sodium, de calcium ou d'ammonium,
   laquelle fraction se lie audit ion métallique et
   ledit complexe contenant de 5 à 1 000 nmol de métal par µmol du composant (b).

2. Un médicament selon la revendication 1 comprenant un complexe dans lequel la quantité de cuivre est de 10 à 1 000 nmol par µmol du composant (b).

3. Un médicament selon les revendications 1 ou 2 comprenant un complexe dans lequel le composant (b) est l'héparine ou une héparine de bas poids moléculaire ou un fragment d'héparine ou un oligosaccharide dérivé de l'héparine.

4. Un médicament selon la revendication 3 comprenant un complexe dans lequel l'oligosaccharide est un tétrasaccharide, un hexasaccharide, un octasaccharide, un décasaccharide, un dodécasaccharide, un tétradécasaccharide ou un hexadécasaccharide.

5. Un médicament selon les revendications 1 ou 2 comprenant un complexe dans lequel le composant (b) est l'héparane-sulfate, un héparane-sulfate de bas poids moléculaire ou des fragments d'héparane-sulfate ou un oligosaccharide dérivé de l'héparane-sulfate.

6. Un médicament selon la revendication 5 comprenant un complexe dans lequel ledit oligosaccharide est un tétrasaccharide, un hexasaccharide, un octasaccharide, un décasaccharide, un dodécasaccharide, un tétradécasaccharide ou un hexadécasaccharide.

7. Un médicament selon l'une quelconque des revendications 1 à 6 comprenant un complexe dans lequel le composant (b) est sous forme d'un sel.

8. Un médicament selon la revendication 7 comprenant un complexe dans lequel le sel est un sel de sodium ou de calcium.

9. Un médicament selon la revendication 8 comprenant un complexe dans lequel le sel est un sel de sodium.

10. Un procédé pour ta préparation d'un complexe selon les revendications 1 à 9 consistant à ajouter de l'héparine, de l'héparane-sulfate, de l'héparine de bas poids moléculaire, de l'héparane-sulfate de bas poids moléculaire, des fragments d'héparine, un fragment d'héparane-sulfate et un oligosaccharide dérivé de l'héparine ou de l'héparane-sulfate à une matrice solide contenant des groupes acides iminodiacétiques auxquels un ion cuivre est lié, séparer la fraction de la matière ajoutée qui n'est pas

liée aux ions cuivre sur la matrice, puis désorber la fraction liée à la matrice.

11. Utilisation d'un complexe de
(a) un ion métallique choisi parmi les ions cuivre, calcium, manganèse, fer et zinc, et
(b) une fraction d'héparine, d'héparane-sulfate, d'héparine de bas poids moléculaire, d'héparane-sulfate de bas poids moléculaire, de fragments d'héparine, de fragments d'héparane-sulfate et d'oligosaccharides dérivés de l'héparine ou de l'héparane-sulfate, ou un sel de ces fractions, lesdites fractions ayant des poids moléculaires de 500 à 35 000 et lesdits sels étant des sels physiologiquement acceptables comme tes sels de sodium, de calcium ou d'ammonium,
laquelle fraction se lie audit ion métallique et
ledit complexe contenant de 5 à 1 000 nmol de métal par µmol du composant (b),
dans la préparation d'un médicament pour le traitement d'une maladie dans laquelle on désire une réduction de l'angiogenèse.

12. Utilisation selon la revendication 11, dans laquelle le médicament comprend le complexe en association avec un composant angiostatique, en particulier ce que l'on appelle un composant angiostatique stéroïdien.

Revendications pour les Etats contractants suivants : ES, GR

1. Un procédé pour la préparation d'un médicament pour le traitement d'une maladie dans laquelle on désire réduire l'angiogenèse, dans lequel le médicament comprend un complexe de
(a) un ion métallique qui est le cuivre et
(b) une fraction d'héparine, d'héparane-sulfate, d'héparine de bas poids moléculaire, d'héparane-sulfate de bas poids moléculaire, de fragments d'héparine, de fragments d'héparane-sulfate et d'oligosaccharides dérivés de l'héparine ou de l'héparane-sulfate, ou un sel de ces fractions, lesdites fractions ayant des poids moléculaires de 500 à 35 000 et lesdits sels étant des sels physiologiquement acceptables comme les sels de sodium, de calcium ou d'ammonium,
laquelle fraction se lie audit ion métallique et
ledit complexe contenant de 5 à 1 000 nmol de métal par µmol du composant (b)
lequel procédé est caractérisé par l'addition de la fraction (b) à une matrice solide contenant des groupes acides iminodiacétiques auxquels un ion cuivre est lié, la séparation de ladite fraction de matière ajoutée qui n'est pas liée aux ions cuivres sur la matrice, puis la désorption de la fraction liée à la matrice.

2. Un procédé selon la revendication 1, dans lequel le médicament comprend un complexe dans lequel la quantité de cuivre est de 10 à 1 000 nmol par µmol du composant (b).

3. Un procédé selon les revendications 1 ou 2, dans lequel le médicament comprend un complexe dans lequel le composant (b) est l'héparine ou une héparine de bas poids moléculaire, ou un fragment d'héparine, ou un oligosaccharide dérivé de l'héparine.

4. Un procédé selon la revendication 3, dans lequel le médicament comprend un complexe dans lequel l'oligosaccharide est un térasaccharide, un hexasaccharide, un octasaccharide, un décasaccharide, un dodécasaccharide, un tétradécasaccharide ou un hexadécasaccharide.

5. Un procédé selon les revendications 1 ou 2, dans lequel le médicament comprend un complexe dans lequel le composant (b) est l'héparane-sulfate, un héparane-sulfate de bas poids moléculaire, ou des fragments d'héparane-sulfate, ou un oligosaccharide dérivé de l'héparane-sulfate.

6. Un procédé selon la revendication 5, dans lequel le médicament comprend un complexe dans lequel ledit oligosaccharide est un tétrasaccharide, un hexasaccharide, un octasaccharide, un décasaccharide, un dodécasaccharide, un tétradécasaccharide ou un hexadécasaccharide.

7. Un procédé selon l'une quelconque des revendications 1 à 6, dans lequel la médicament compr nd un complexe dans lequel le composant (b) est sous forme d'un sel.

8. Un procédé selon la revendication 7, dans lequel le médicament comprend un complexe dans lequel le sel est un sel de sodium ou de calcium.

9. Un procédé selon la revendication 8, dans lequel le médicament comprend un complexe dans lequel le sel est un sel de sodium.

10. Utilisation d'un complexe de

(a) un ion métallique choisi parmi les ions cuivre, calcium, manganèse, fer et zinc, et

(b) une fraction d'héparine, d'héparane-sulfate, d'héparine de bas poids moléculaire, d'héparane-sulfate de bas poids moléculaire, de fragments d'héparine, de fragments d'héparane-sulfate et d'oligosaccharides dérivés de l'héparine ou de l'héparane-sulfate, ou un sel de ces fractions, lesdites fractions ayant des poids moléculaires de 500 à 35 000 et lesdits sels étant des sels physiologiquement acceptables comme les sels de sodium, de calcium ou d'ammonium,

laquelle fraction se lie audit ion métallique et

ledit complexe contenant de 5 à 1 000 nmol de métal par µmol du composant (b),

dans la préparation d'un médicament pour le traitement d'une maladie dans laquelle on désire une réduction de l'angiogenèse.

11. Utilisation selon la revendication 10, dans laquelle le médicament comprend le complexe en association avec un composant angiostatique, en particulier ce que l'on appelle un composant angiostatique stéroïdien.

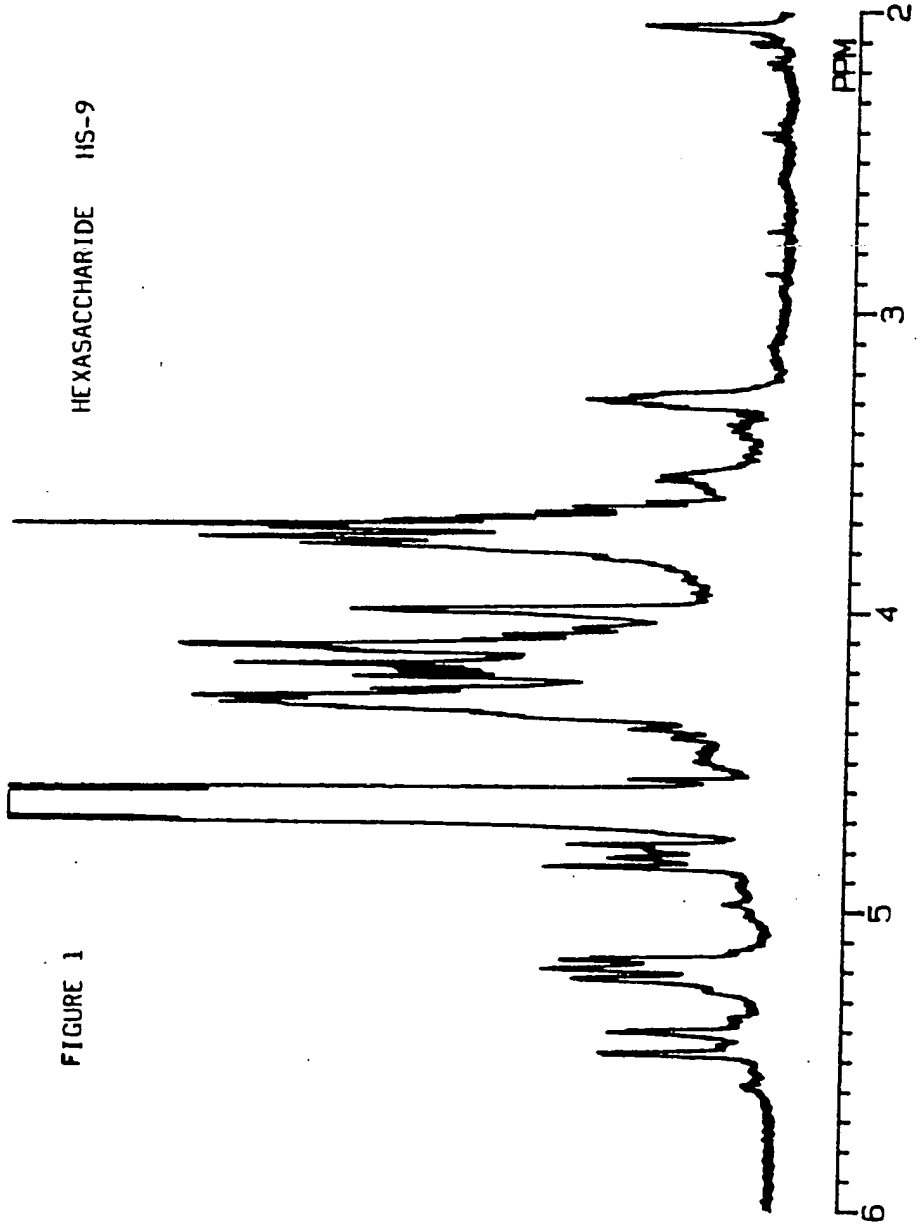

FIGURE 1